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Regulation of Rat Hepatic Delta-Aminolevulinic Acid Synthetase and Heme Oxygenase Activities: Evidence for Control by Heme and Against Mediation by Prosthetic Iron

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16. ABSTRACT

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REGULATION OF RAT HEPATIC δ-AMINOLEVULINIC ACID SYNTHETASE AND HEME OXYGENASE ACTIVITIES: EVIDENCE FOR CONTROL BY HEME AND AGAINST MEDIATION BY PROSTHETIC IRON

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Abstract—1. The effects of *in vivo* administration of 6 compounds on the activity of δ-aminolevulinic acid (ALA) synthetase and heme oxygenase were determined.

2. The order of decreasing potency in reducing ALA synthetase activity was heme, bilirubin, protoporphyrin IX, bilirubin dimethyl ester, $CoCl_2$ and $FeCl_3$.

3. The chelating agents EDTA and deferoxamine did not prevent heme's repression of ALA synthetase or induction of heme oxygenase activity.

4. The dose response, time course, enzyme subcellular distribution and chelation antagonism studies all suggest that heme itself, and not iron, regulates the rate limiting enzymatic steps of rat hepatic heme synthesis and degradation.

INTRODUCTION

The control of hepatic heme levels is important because heme is required to unite with specific apocytochromes of the P-450 class to form functional "drug metabolizing enzymes" (Correia & Meyer 1975; Kitchin & Woods 1978; Farrell *et al.*, 1981). In mouse liver, 40% of the synthesized heme is required for cytochrome P-450 synthesis (Wada *et al.*, 1968). Heme has also been found to induce cyclic, out of phase progressively dampened oscillations of ALA synthetase (EC 2.3.1.37) and HO (EC 1.14.99.3) activity (Waxman *et al.*, 1966; Schacter *et al.*, 1976). Granick (1966) first proposed that heme controlled the rate of its own synthesis and hence hepatic heme levels, by transcriptional feedback repression of ALA synthetase, the first and rate limiting enzyme in this pathway (Model **I** of Fig. 1). Tyrell & Marks (1972), working with chick embryo liver culture, suggested that this feedback repression was translational in nature. Control of ALA synthetase by heme has been proposed to act via (i) transcription (Granick, 1966), (ii) enzyme inhibition (Scholnick *et al.*, 1969; Whiting & Elliot, 1972) and (iii) inhibited enzyme transport into the mitochondrion (Hayashi *et al.*, 1972). Not only heme, but many metals ions (e.g. Cd, Se, Ag, Cu, Co, Ni, Mn, Fe, Pb) can induce hepatic heme oxygenase activity (Eaton *et al.*, 1980). This induction appears to be a non-specific effect, as metal ions which do not form chelates with porphyrins, which are not nutritionally required for normal growth, and which are not found in high concentrations in the body all can induce HO activity. This leads to a second possible model of the control of hepatic heme synthesis and levels; any extra heme is catabolized to iron which induces HO. This HO induction decreases hepatic heme levels and derepression of ALA synthetase can thus restore hepatic heme homeostatis (Model **II** of Fig. 1). A third proposed mechanism is that iron, released by HO catabolism of heme, directly feedback represses ALA synthetase activities (Model **III** of Fig. 1) (Maines & Kappas, 1978).

In this study, experiments were performed to determine which of the 3 proposed models of the regulation of hepatic heme levels best represents this pathway *in vivo*. Dose response, time course and subcellular distribution studies of ALA synthetase activity were performed after administration of protoporphyrin IX, heme, bilirubin, bilirubin dimethyl ester, cobalt and iron to male rats. Further experiments were performed with EDTA and deferoxamine to determine if these chelating agents could antagonize heme's repression of ALA synthetase and induction of HO.

MATERIALS AND METHODS

Materials

Hemin chloride was purchased from Calbiochem. Protoporphyrin IX, bilirubin and bilirubin dimethyl ester were obtained from Porphyrin Products. Cobaltous chloride and ferric chloride were purchased from K & K Laboratories. Allylisopropylacetamide and deferoxamine mesylate (Desferal) were generous gifts of Hoffman La Roche, Inc. and Ciba Pharmaceuticals Co., respectively. Succinyl-CoA

The abbreviations used are ALA, δ-aminolevulinic acid; HO, heme oxygenase; AIA, allyisopropylacetamide; EDTA, disodium ethylenediamine tetraacetic acid.

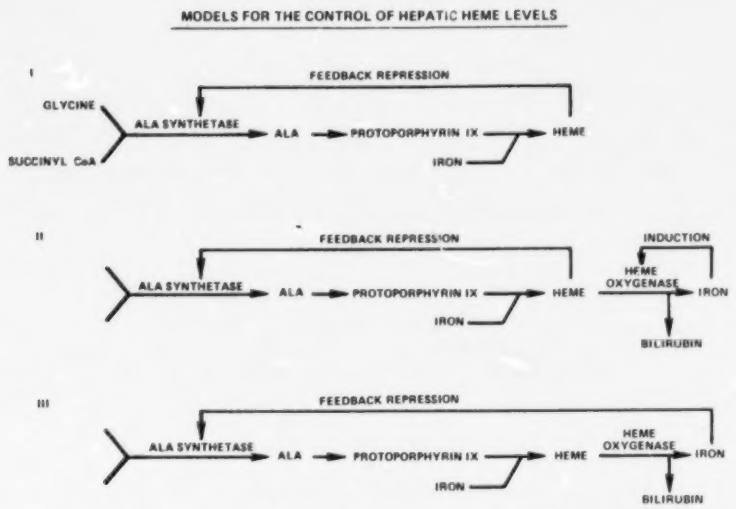

Fig. 1. Three proposed models for the control of hepatic heme levels. In Model **I**, heme is a direct feedback repressor on ALA synthetase. Model **II** postulates that iron released from hepatic heme induces heme oxygenase activity, lowers hepatic heme levels, and thus derepresses ALA synthetase activity via the mechanism proposed in Model **I**. In both Model **I** and **II** an excess of cellular heme represses ALA synthetase and a shortage of cellular heme causes derepression of ALA synthetase. Model **III** hypothesizes that iron directly acts as a feedback repressor of ALA synthetase.

synthetase (succinic thiokinase, EC 6.2.1.4), pyridoxal-5-phosphate, adenosine 5′-triphosphate, guanosine 5′-triphosphate, coenzyme A, dithioerythritol, δ-aminolevulinic acid, dextran and NADPH were obtained from Sigma Chemical Co. Fisher Sci. Co. supplied the disodium EDTA, glycine, sodium succinate and *p*-dimethylaminobenzaldehyde. Other chemicals were of reagent grade and were obtained from standard commercial sources.

Treatment of animals and preparation of subcellular fractions

Male Sprague–Dawley rats (CD strain) (120–200 g), obtained from Charles River Laboratories, were housed 4 per plastic cage with food (Purina Rat Chow) and water *ad libitum*. Rats were starved 24 hr before the administration of any compound and animals were deprived of food until sacrifice. Protoporphyrin IX and hemin chloride were dissolved in 0.1 N NaOH, titrated to pH 7.5 with 0.1 N HCl and administered intravenously to rats via the tail vein. Bilirubin and bilirubin dimethyl ester were complexed with 0.5% human serum albumin in 0.9% saline with the use of ultrasound (Heat Systems Model W-220 F) and administered to rats in the tail vein. Cobaltous chloride and ferric chloride were dissolved in 0.9% saline containing a 5 to 1 weight ratio of dextran per metal chloride and injected subcutaneously in the neck region. In antagonism studies, EDTA and deferoxamine mesylate were dissolved in 0.9% saline at pH 7.5 and injected intraperitoneally 4 and 2 hr before sacrifice. Allylisopropylacetamide was dissolved in 50% ethyl alcohol and administered subcutaneously. Administration of the vehicles saline, dextran in saline, human serum albumin in saline and 50% ethyl alcohol did not affect hepatic ALA synthetase or HO activities. With the exception of EDTA, all drugs were prepared fresh just before use. Preparation of hepatic mitochondrial and post mitochondrial supernatant fractions were performed as described by Kitchin & Woods (1978).

Assay of enzyme activities

ALA synthetase activity was determined by the method of Scholnick *et al.* (1972) using a succinyl CoA generating system with the modifications that β-mercaptoethanol was replaced by 0.1 mM dithioerythritol and that 0.1 mM guanosine 5′-triphosphate (for the succinyl CoA generating system) was included in the incubation media. Enzymatic formation of ALA at 37°C during the 30 min incubation period was determined spectrophotometrically at 553 nm by using a molar extinction coefficient of 5.3×10^4 (Woods, 1974). Post-mitochondrial supernatant HO activities were determined by using 20 μM hemin chloride freshly dissolved in 0.1 N NaOH and titrated to pH 7.4 as the substrate. Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin (fraction V) as a standard. Data were analyzed for statistical significance by analysis of variance and the Student's *t*-test (Snedecor & Cochran, 1967).

RESULTS

Dose response relationship

As an initial approach to determining likely regulators of rat hepatic heme synthesis and degradation, the dose response relationships for 6 compounds on mitochondrial ALA synthetase (Fig. 2) and post-mitochondrial supernatant HO activities were determined. Intravenous administration of hemin significantly reduced ($P < 0.05$) ALA synthetase activities by approx. 60% at 1.22, 3.24, 12.2 and 32.4 μmol/kg. Administration of iron III/dextran *in vivo* produced either no effect or an elevation in the *in vitro* hepatic mitochondrial ALA synthetase activity. Subcutaneous injection of $CoCl_2$/dextran eliminated ($P < 0.001$) mitochondrial ALA synthetase activity at the high doses of 122 and 324 μmol/kg. Protoporphyrin IX reduced mitochondrial ALA synthetase activities ($P < 0.05$) at 12.2 and 32.4 μmol/kg. Administration of 32.4 μmol/kg bilirubin or bilirubin dimethyl ester both significantly reduced ALA synthetase activity.

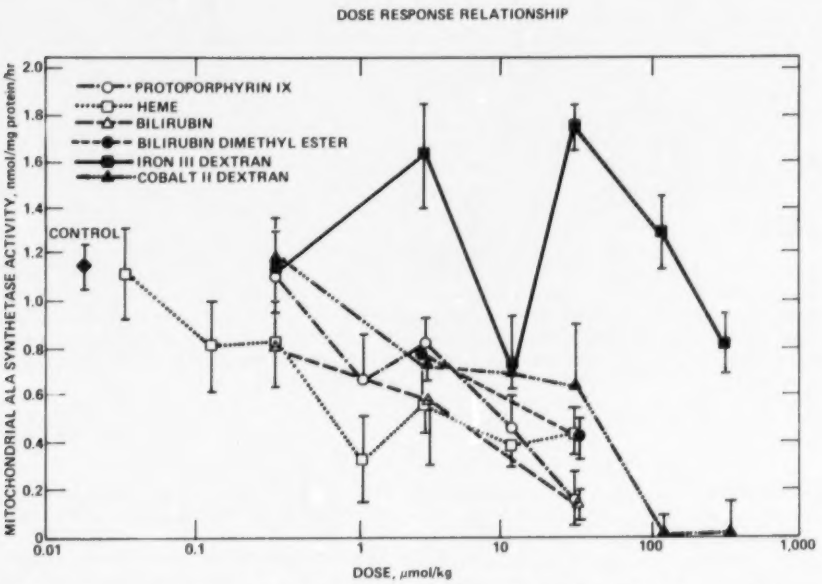

Fig. 2. Dose response relationship for the repression of ALA synthetase. The indicated doses of these six compounds were administered to rats *in vivo* 4 hr before sacrifice and the resulting mitochondrial ALA synthetase activity determined *in vitro* as described in Materials and Methods. Data is presented as the mean ± SEM for five or more determinations.

With respect to hepatic heme oxygenase activity, administration of either iron/dextran, protoporphyrin IX, bilirubin, or bilirubin dimethyl ester did not affect the enzyme activity (Fig. 3). Administration of 12.2 or 32.4 μmol/kg of hemin induced HO activities by 4.6- and 8.9-fold, while cobalt chloride in the 10-fold higher doses of 122 and 324 μmol/kg induced HO activity by 1.5- and 3.9-fold, respectively.

Time course studies of the effects of protoporphyrin IX, heme, bilirubin, bilirubin dimethyl ester, cobalt and iron on rat hepatic ALA synthetase and HO activities

After the completion of dose response experiments, doses of 32.4 μmol/kg of the tetrapyrroles and 324 μmol/kg of the metals were used in time course studies. Hemin treatment repressed ALA synthetase

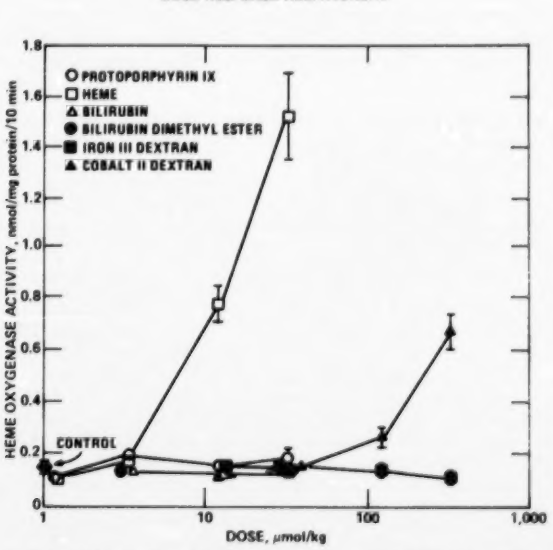

Fig. 3. Dose response relationship for the induction of heme oxygenase activity. The indicated doses of these six compounds were administered to rats 4 hr before sacrifice and the resulting hepatic post mitochondrial supernatant heme oxygenase activities determined *in vitro* as described in Materials and Methods. Data is presented as the mean ± SEM for five or more determinations.

Table 1. Time course study of the effect of tetrapyrroles and metals on ALA synthetase and heme oxygenase activity

	ALA synthetase activity, nmol/mg protein per hr			Heme oxygenase activity, nmol/mg protein per 10 min		
	4 hr	12 hr	24 hr	4 hr	12 hr	24 hr
Saline	1.15 ± 0.17	1.21 ± 0.18	1.37 ± 0.13	0.13 ± 0.008	0.16 ± 0.016	0.17 ± 0.046
Protoporphyrin IX	0.17 ± 0.11†	0.52 ± 0.15†	0.70 ± 0.25*	0.14 ± 0.014	0.15 ± 0.014	0.21 ± 0.030
Heme	0.50 ± 0.10*	2.04 ± 0.66	2.23 ± 0.43*	1.52 ± 0.17‡	1.14 ± 0.17‡	0.68 ± 0.046‡
Bilirubin	0.14 ± 0.07‡	1.21 ± 0.29	1.71 ± 0.30	0.14 ± 0.013	0.16 ± 0.011	0.14 ± 0.038
Bilirubin dimethyl ester	0.42 ± 0.08†	1.46 ± 0.21	1.79 ± 0.22	0.14 ± 0.015	0.17 ± 0.023	0.13 ± 0.023
Iron/dextran	0.82 ± 0.13	0.98 ± 0.16	1.19 ± 0.28	0.10 ± 0.015	0.13 ± 0.005	0.22 ± 0.031
Cobalt/dextran	0.02 ± 0.14‡	1.05 ± 0.22	1.22 ± 0.31	0.67 ± 0.068‡	1.97 ± 0.10‡	2.17 ± 0.23‡

Tetrapyrroles (32.4 μmol/kg) or metals (324 μmol/kg) were administered to male rats at various times before sacrifice. The resulting mitochondrial ALA synthetase and heme oxygenase enzyme activities were determined as described in Materials and Methods. Data is the mean ± SEM for 5 or more determinations.

* $P < 0.05$.
† $P < 0.01$.
‡ $P < 0.001$.

activity and caused a "rebound effect," an eventual increase in enzyme activity by 12 hr after treatment (Table 1). Subcutaneous injection of iron/dextran did not effect ALA synthetase activity at any of the three time points examined. Administration of bilirubin, bilirubin dimethyl ester and cobalt/dextran caused decreases in ALA synthetase at the 4 hr time point, essentially no effect at 12 hr, and a suggestion of a possible ALA synthetase "rebound effect" for bilirubin and bilirubin dimethyl ester at 24 hr. ALA synthetase activities were lowered by protoporphyrin IX administration 4, 12 and 24 hr before sacrifice.

Administration of iron/dextran, protoporphyrin IX, bilirubin and bilirubin dimethyl ester 4, 12 and 24 hr before sacrifice did not affect rat hepatic post-mitochondrial supernatant HO activity. Intravenous heme treatment induced HO activity from a control level of 0.13 to 1.52, 1.14 and 0.68 nmol bilirubin/mg protein per 10 min at 4, 12 and 24 hr after sacrifice. Subcutaneous administration of cobalt/dextran induced HO activity more slowly, reaching values of 0.67, 1.97 and 2.17, 4, 12 and 24 hr after treatment. The induction of HO activity by heme at 4 hr was blocked by administration of cycloheximide (data not shown).

Effect of tetrapyrroles and metals on the subcellular distribution of allylisopropylacetamide-induced ALA synthetase

Since heme is known to inhibit the transport of the protein ALA synthetase from the cytosol into the mitochondria (Hayashi *et al.*, 1972), its enzymatic site of action, the effects of the 6 compounds on the subcellular distribution of ALA synthetase was determined. In AIA pretreated rats, heme reduced mitochondrial ($P < 0.05$), and did not affect either the post-mitochondrial supernatant or the total hepatic ALA synthetase activity (Table 2). Two hours after intravenous administration, however, heme not only decreased mitochondrial ALA synthetase from 8.40 to 3.05 nmol of ALA/mg per hr but also increased post-mitochondrial supernatant and total enzyme activity to 18.5 and 1360 nmol ALA/g liver per hr. Although iron/dextran treatment did not markedly affect the subcellular distribution of ALA synthetase, cobalt/

Table 2. Effect of tetrapyrroles and metals on the subcellular distribution of induced ALA synthetase activity

Treatment	Mitochondrial ALA synthetase (nmol/mg protein per hr)	Post mitochondrial supernatant ALA synthetase (nmol/mg protein per hr)	Total ALA synthetase (nmol/g liver per hr)
AIA	8.40 ± 1.35	3.55 ± 0.37	353 ± 41
AIA + protoporphyrin IX	4.76 ± 0.68‡	2.46 ± 0.47†	232 ± 43‡
AIA + heme*	2.72 ± 0.47§	3.96 ± 0.54	286 ± 44
AIA + bilirubin	6.38 ± 1.14	2.47 ± 0.45†	252 ± 46‡
AIA + bilirubin dimethyl ester	5.95 ± 1.20	2.61 ± 0.43†	257 ± 40†
AIA + Fe/dextran	6.44 ± 0.91	3.25 ± 0.26	317 ± 20
AIA + Co/dextran	2.64 ± 0.35§	20.5 ± 4.9§	1320 ± 241§

Starved male rats were given 300 mg/kg of AIA subcutaneously 16 hr before sacrifice. Four hours before sacrifice tetrapyrroles (32.4 μmol/kg, i.v.) or metals (324 μmol/kg, s.c.) were also administered. ALA synthetase enzyme activities were determined in mitochondrial fraction or in post-mitochondrial supernatant fractions. The two specific activities multiplied by the protein content of the fraction gives the total ALA synthetase activity. Data are the mean ± SEM for 6 or more determinations.

* When heme was administered 2 hr before sacrifice to AIA pretreated rats, ALA synthetase activities were 3.65 ± 0.65, 18.5 ± 5.2 and 1360 ± 380 in the mitochondrial, post-mitochondrial supernatant and total liver fractions (excluding the nuclear pellet), respectively.
† $P < 0.05$.
‡ $P < 0.01$.
§ $P < 0.001$.

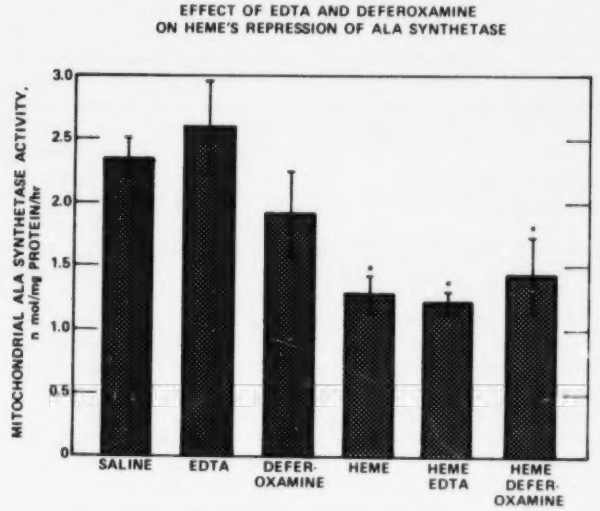

Fig. 4. Effect of EDTA and deferoxamine on heme's repression of hepatic mitochondrial ALA synthetase activity. Heme (12.2 μmol/kg given i.v. 4 hr before sacrifice), EDTA (24.5 μmol/kg given i.p. both 2 and 4 hr before sacrifice) or deferoxamine (13.5 μmol/kg given i.p. 2 and 4 hr before sacrifice) were administered to rats either alone or in combination. The resulting ALA synthetase activities were determined *in vitro* as described in Materials and Methods. The mean ± SEM for six or more determinations is presented. An asterisk indicates $P < 0.05$ vs saline treated controls.

dextran significantly reduced ($P < 0.05$) mitochondrial ALA synthetase, and caused a large increase in post-mitochondrial supernatant and total ALA synthetase activity.

Effect of chelating agents on heme's repression of hepatic ALA synthetase and induction of HO activity

Iron has been postulated to be the regulator of hepatic ALA synthetase (Maines & Kappas, 1978) by one group who interprets heme as an iron carrier. For this reason, heme treatment was combined with EDTA (4-fold molar excess) or deferoxamine (2.2-fold molar excess) administration to chelate any iron released by metabolism of heme by HO *in vivo*. Neither EDTA nor deferoxamine administration affected hepatic mitochondrial ALA synthetase (Fig. 4) or post mito-

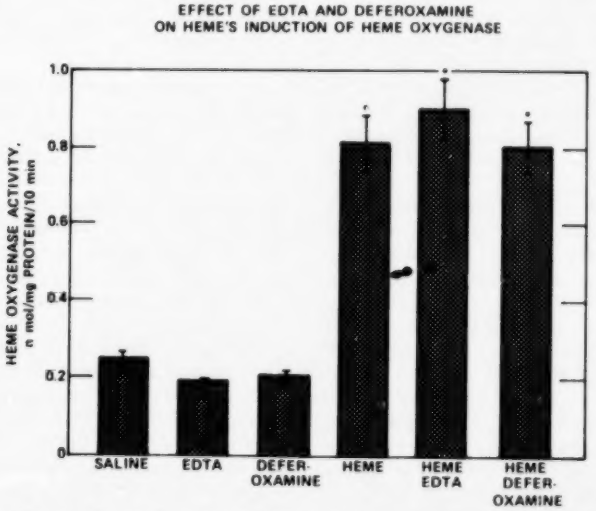

Fig. 5. Effect of EDTA and deferoxamine on heme's induction of hepatic post mitochondrial heme oxygenase activity. Heme (12.2 μmol/kg given i.v. 4 hr before sacrifice), EDTA (24.5 μmol/kg given i.p. both 2 and 4 hr before sacrifice) or deferoxamine (13.5 μmol/kg given i.p. both 2 and 4 hr before sacrifice) were administered to rats either alone or in combination. The resulting post-mitochondrial supernatant heme oxygenase enzyme activities were determined *in vitro* as described in Materials and Methods. Data presented is the mean ± SEM for six or more determinations. An asterisk indicates $P < 0.001$ vs saline treated controls.

chondrial supernatant HO activities (Fig. 5) *per se*. Heme (12.2 μmol/kg) significantly repressed ALA synthetase activity by about 45%; this effect was not antagonized by the chelators EDTA or deferoxamine. Heme treatment induced HO activity about 5-fold; again neither EDTA nor deferoxamine prevented this induction.

DISCUSSION

Heme in doses as low as 100–150 μg per rat reduced ALA synthetase activity, the rate limiting enzyme in heme synthesis, by about 60%. This finding is consistent with Model **I** or **II**, but not Model **III** (Fig. 1). This low, physiologically relevant dose of exogenous heme, is within one order of magnitude of the 10–100 nmol concentration of "free regulatory heme" proposed by Granick & Beale (1978). Iron/dextran, in molar doses up to 1000 times larger, did not reduce hepatic ALA synthetase activity. In other studies, ferric citrate has been shown to increase hepatic ALA synthetase activities as well as potentiate the induction of ALA synthetase by AIA (Stein *et al.*, 1970). This experimental evidence argues against the hypothesis that heme's control of ALA synthetase is mediated via iron (Model **III**).

Iron/dextran did not induce HO in this study, although other investigators were able to induce HO 3- and 6-fold with 750 mg/kg (given over 6 weeks) or 125 mg/kg of iron/dextran, respectively (Ibrahim *et al.*, 1979). These enormous doses of iron are 379 and 63 times the molar dose of heme necessary to induce HO.

In the study presented here heme oxygenase activity was induced by only cobalt chloride and heme. Heme was about 25 times more potent than $CoCl_2$. The dose response relationships for $CoCl_2$ induction of HO 4 hr after administration is in rough agreement with the studies performed by Woods & Carver (1977) in fetal rat liver and by Maines & Kappas (1977b) in adult animals (both studies were 24 hr after $CoCl_2$ administration). The failure of iron/dextran to induce HO activity in this study and the high doses required in other investigations argue against Model **II** of Fig. 1. As only 1/10 as much heme is needed to repress ALA synthetase as is needed to induce HO, this suggests that heme may primarily regulate hepatic heme levels by regulating the heme synthetic pathway. At very high hepatic heme levels, HO induction would also help restore hepatic heme homeostasis.

If heme acted as an "iron carrier" to efficiently transport iron to regulatory sites within the hepatocytes, it would be expected that chelators such as EDTA or deferoxamine could antagonize heme's repression of mitochondrial ALA synthetase and induction of HO. This study indicated that administration of these two chelators do not antagonize heme's regulatory effects. Moreover, in this study neither EDTA nor deferoxamine administration *per se* raised ALA synthetase or lowered HO activities arguing against control of the heme pathway via endogenous iron. Maines & Kappas (1978) also have observed that neither EDTA nor dihydroxybenzoic acid treatment induced ALA synthetase in adult rats. Other studies have shown deferoxamine increases HO activity but does not increase ALA synthetase (Liem *et al.*, 1979). These experimental findings are incompatible with Models **II** and **III**.

The half life of rat hepatic ALA synthetase has been estimated to be between 35 and 70 min (Marver *et al.*, 1966; Hayashi *et al.*, 1969; Hayashi *et al.*, 1980a) while the half life of hepatic heme oxygenase is over 15 hr (Maines and Kappas, 1977a). The difference between the half lines of these two enzymes suggest that a finer temporal control of hepatic heme levels could be achieved by regulating ALA synthetase rather than HO. Heme administration caused an initial decrease and an eventual increase in ALA synthetase activity in the 24 hr time course study. During this time HO activity were increased but had not yet returned to the original control level. This observation would favor Model **I** and argue against Model **II**.

It is recognized that the pharmacokinetics of subcutaneously administered Fe/dextran and Co/dextran are quite different from that of intravenously administered heme, protoporphyrin IX, bilirubin and bilirubin dimethyl ester. The differences in distribution and sequestration of the 6 compounds can effect the dose response and time course relationships. Hemin is known to be bound and transported to the liver by a specific serum protein, hemopexin (Muller-Eberhard & Morgan, 1975). Protoporphyrin IX can also bind to hemopexin, but with a much lower affinity (Seery & Muller-Eberhard, 1973).

In this study, administration of either heme (2 hr before sacrifice) or cobalt/dextran (4 hr before sacrifice) to AIA-pretreated rats (16 hr before sacrifice) increased the total hepatic ALA synthetase per gram liver by about 3.8-fold. This finding is in contrast to the results of Hayashi *et al.* (1972, 1980b) who observed that the sum of mitochondrial and post mitochondrial supernatant ALA synthetase in AIA or 3,5-dicarbethoxy-1,4-dihydrocollidine never exceeded to total hepatic ALA synthetase activity in rats given either inducer alone.

To summarize, previous evidence for heme regulation of its own synthesis and degradation which have been confirmed in this study are: (1) heme decreases ALA synthetase activity; (2) heme causes a rebound effect (an eventual overshooting of ALA synthetase activity following the initial decrease) in ALA synthetase; (3) heme induces heme oxygenase activity; and (4) heme inhibits transport of ALA synthetase into mitochondria. Results of the experiments presented here argue against iron's control of hepatic heme because: (1) exogenous heme, in doses as low as 1.22 μmol/kg (100–150 μg heme/rat), can decrease ALA synthetase activity; (2) iron does not decrease ALA synthetase activity; (3) iron in intermediate doses does not induce HO activity; (4) chelators do not increase ALA synthetase activities; and (5) chelators do not antagonize heme's repression of ALA synthetase or induction of HO. Thus, it is concluded that heme, not iron, regulates the hepatic heme levels, principally by controlling hepatic mitochondrial ALA synthetase activity (Model **I** of Fig. 1).

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